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## CHAPTER II

**TRANSMITTAL LETTER  
TO THE UNITED STATES ELECTED OFFICE (EO/US)  
(ENTRY INTO U.S. NATIONAL PHASE UNDER CHAPTER II)**

<b>PCT/US97/23927</b>	<b>19 December 1997 (19.12.97)</b>	<b>27 December 1996 (27.12.96)</b>
International Application Number	International Filing Date	International Earliest Priority Date

**TITLE OF INVENTION: G-rich Oligo Aptamers and Methods of Modulating an Immune Response**

**APPLICANT(S): ICN Pharmaceuticals, Inc.; TAM, Robert**

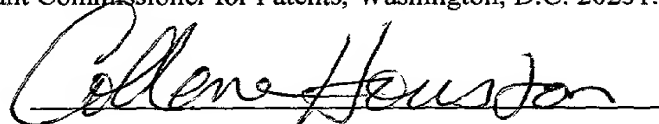
**Box PCT  
Assistant Commissioner for Patents  
Washington D.C. 20231  
ATTENTION: EO/US**

1. Applicant herewith submits to the United States Elected Office (EO/US) the following items under 35 U.S.C. § 371:
  - a. This express request to immediately begin national examination procedures (35 U.S.C. § 371(f)).
  - b. The U.S. National Fee (35 U.S.C. § 371(c)(1)) and other fees (37 C.F.R. § 1.492) as indicated below:

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**CERTIFICATION UNDER 37 C.F.R. 1.10\***

I hereby certify that this correspondence and the documents referred to as attached therein are being deposited with the United States Postal Service on this date 6/16/99, in an envelope as Express Mail Post Office to Addressee, Mailing Label Number EL315366023US, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.



Collene Houston

## 2. Fees

CLAIMS FEE	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
	TOTAL CLAIMS	25 - 20 =	5	x \$18.00 =	\$90.00
	INDEPENDENT CLAIMS	1 - 3 =	0	x \$78.00 =	\$0.00
	MULTIPLE DEPENDENT CLAIM(S) (if applicable) + \$260.00				\$0.00
BASIC FEE	U.S. PTO WAS INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where an International preliminary examination fee as set forth in § 1.482 has been paid on the international application to the U.S. PTO: and the international preliminary examination report states that the criteria of novelty, inventive step (non-obviousness) and industrial activity, as defined in PCT Article 33(2) to (4) have been satisfied for all the claims presented in the application entering the national stage (37 C.F.R. § 1.492(a)(4)) ..... \$96.00				\$96.00
	Total of above Calculations				= \$186.00
SMALL ENTITY	Reduction by 50% for filing by small entity, if applicable. Affidavit must be filed. (note 37 CFR §§ 1.9, 1.27, 1.28)				- \$0.00
	Subtotal				\$186.00
	Total National Fee				\$186.00
	Fee for recording the enclosed assignment document \$40.00 (37 C.F.R. § 1.21(h)). See attached ASSIGNMENT COVER SHEET.				\$0.00
TOTAL	Total Fees enclosed				\$186.00

A check in the amount of \$186.00 to cover the above fees is enclosed.

3. A copy of the International application as filed (35 U.S.C. § 371(c)(2)) is not required, as the application was filed with the United States Receiving Office.
4. A translation of the International application into the English language (35 U.S.C. § 371(c)(2)) is not required as the application was filed in English.
5. Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. § 371(c)(3)) have been transmitted by the International Bureau.  
Date of mailing of the amendment (from form PCT/IB/308): 9 July 1998

6. A translation of the amendments to the claims under PCT Article 19 (38 U.S.C. § 371(c)(3)) is not required as the amendments were made in the English language.
7. A copy of the international examination report (PCT/IPEA/409) is not required as the application was filed with the United States Receiving Office.
8. Annex(es) to the international preliminary examination report is/are not required as the application was filed with the United States Receiving Office.
9. A translation of the annexes to the international preliminary examination report is not required as the annexes are in the English language,
10. An oath or declaration of the inventor (35 U.S.C. § 371(c)(4)) complying with 35 U.S.C. § 115 will follow.
11. An International Search Report (PCT/ISA/210) or Declaration under PCT Article 17(2)(a) is not required, as the application was searched by the United States International Searching Authority.
12. Additional documents:
  - a. Preliminary amendment (37 C.F.R. § 1.121)
  - b. Check No. 7675 for \$186 and Return Receipt Postcard
13. The above items are being transmitted before 30 months from any claimed priority date.

### AUTHORIZATION TO CHARGE ADDITIONAL FEES

The Commissioner is hereby authorized to charge the following additional fees that may be required by this paper and during the entire pendency of this application to Account No.: 500341

37 C.F.R. § 1.492(a)(1), (2), (3), and (4) (filing fees)  
37 C.F.R. § 1.492(b), (c), and (d) (presentation of extra claims)  
37 C.F.R. § 1.17 (application processing fees)  
37 C.F.R. §§ 1.17(a)(1)-(5) (extension fees pursuant to 1.136(a))

Respectfully submitted,  
Crockett and Fish

Dated: June 16, 1999

By: 

Robert D. Fish  
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE  
WASHINGTON, D.C. 20231

Inventor: **Robert Tam**

Examiner: **Not Assigned**

Serial No: **Not Assigned**

Art Unit: **Not Assigned**

Filed: **Herewith**

For: **G-rich Oligo Aptamers and Methods of  
Modulating an Immune Response**

**PRELIMINARY AMENDMENT**

The Honorable Commissioner  
of Patents and Trademarks  
Washington, D.C. 20231

Dear Sir:

Please enter the following as a preliminary amendment.

**IN THE CLAIMS**

1. (amended) An aptamer having a length of between about 12 and 22 nucleic acid units, inclusive, and a sequence which includes at least two G-rich regions selected from the group consisting of GGnG, GGGG, GnGG, nGGG and GGGn, where G is guanidine and n is any nucleotide.

Claim 2, replace "less than seven" with --by two to seven--.

Claim 7, replace "less than seven" with --by two to seven--.

Claim 8, replace "less than seven" with --by two to seven--.

Claim 9, replace "less than seven" with --by two to seven--.

Claim 10, replace "less than seven" with --by two to seven--.

Claim 17, replace "any of claims 1-16" with --claim 1--.

Claim 18, replace "any of claims 1-16" with --claim 1--.

IN THE SPECIFICATION

Page 4, line 11, replace "(**patent CD28 Tam**)" with "(See PCT/US96/01507, filed August 30, 1996)".

Page 5, replace the first paragraph under the heading "SUMMARY OF THE INVENTION" with the following paragraph:

The present invention provides aptamers having a length of between about 12 and 22 nucleic acid units, inclusive, and a sequence which includes at least two G-rich regions selected from the group consisting of GGnG, GGGG, GnGG, nGGG and GGGn, where G is guanidine and n is any nucleotide.

Page 5, line 24, delete the words "are provided" at the end of the first sentence.

Page 7, line 3, replace "which" with "that".

Page 7, line 29, insert "and" before "therefore do not encounter . . ."

Page 9, line 15, replace the sentence which begins, "Equipment for such synthesis . . ." with the following sentence:

Several vendors including Applied Biosystems sell equipment for such synthesis.

Page 9, line 34, replace "less" with "fewer".

Page 9, line 35, delete "A" and replace "segment" with "segments".

Page 9, line 36, replace "is" with "are".

Page 18, line 16, replace "thus inhibit" with "which inhibits".

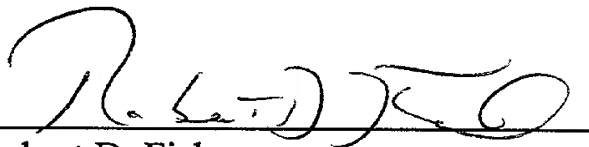
Page 18, line 17, replace "decrease" with "decreases".

REMARKS

The requested changes are either typographical errors or are intended to more clearly delineate the subject matter. No new matter has been added.

Respectfully submitted,  
Crockett and Fish

Dated: 6/16/99

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G-RICH OLIGO APTAMERS AND METHODS OF  
MODULATING AN IMMUNE RESPONSE

5 FIELD OF THE INVENTION

The field of the invention is immunology.

BACKGROUND OF THE INVENTION

10 The pathogenesis and exacerbation of many prevalent T-cell mediated diseases result from an inappropriate immune response driven by abnormal T-cell activation. A number of other diseases are thought to be caused by aberrant T-cell activation including Type I (insulin-dependent) diabetes mellitus, thyroiditis, sarcoidosis, multiple sclerosis, autoimmune uveitis, rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease (Crohn's and ulcerative colitis) and aplastic anemia. In addition, a variety of syndromes including septic shock and tumor-induced cachexia may involve T-cell activation and augmented production of potentially toxic levels of lymphokines. Normal T-cell activation also mediates the rejection of transplanted cells and organs by providing the necessary signals for the effective destruction of the "foreign" donor tissue.

15 The activation of T-lymphocytes leading to T-cell proliferation and gene expression and secretion of specific immunomodulatory cytokines requires two independent signals. The first signal involves the recognition, by specific T-cell receptor/CD3 complex, of antigen presented by major histocompatibility complex molecules on the surface of antigen-presenting cells (APCs). Antigen-nonspecific intercellular interactions between T-cells and APCs provide the second signal which serves to regulate T-cell responses to antigen. These secondary or costimulatory signals determine the magnitude of a T-cell response to antigen. Costimulated cells react by increasing the levels of specific cytokine gene transcription and by stabilizing selected mRNAs. T-cell activation in the absence of costimulation results in an aborted or anergic T-cell response. One key costimulatory signal is provided by interaction of the T-cell surface receptor CD28 with B7-related molecules on APC (Linsley and Ledbetter (1993) *Annu Rev Immunol* 11 : 191-212). CD28 is constitutively expressed on 95% of CD4<sup>+</sup> T-cells (which provide helper functions for B-cell antibody production) and 50% of CD8<sup>+</sup> T-cells (which have cytotoxic functions) (Yamada et al (1985) *Eur J Immunol* 15 : 1164-1168). Following antigenic or *in vitro* mitogenic stimulation, further induction of surface levels of CD28 occurs, as well as the production of certain immunomodulatory cytokines. These include interleukin-2 (IL-2), required for cell cycle progression of T-cells, interferon-gamma (IFN $\gamma$ ), which displays a wide variety of anti-viral

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and anti-tumor effects and interleukin-8 (IL-8), known as a potent chemotactic factor for neutrophils and lymphocytes. These cytokines have been shown to be regulated by the CD28 pathway of T-cell activation (Fraser et al (1991) *Science* 251 : 313-316, Seder et al (1994) *J Exp Med* 179 : 299-304, Wechsler et al (1994) *J Immunol* 153 : 2515-2523). IL-2, IFN $\gamma$  and IL-8 are essential in promoting a wide range of immune responses and have been shown to be overexpressed in many T-cell mediated disease states.

In psoriasis, activated lesional T-cells predominantly release Th1 cytokines such as IL-2 and IFN $\gamma$  (Schlaak et al (1994) *J Invest Derm* 102 : 145-149). These secreted cytokines induce normal keratinocytes to express the same phenotype (HLA DR<sup>+</sup>/ICAM-1<sup>+</sup>) as found in psoriasis lesions (Baadsgaard et al (1990) *J Invest Derm* 95 : 275-282). Also IL-8, by virtue of its *in vitro* and *in vivo* proinflammatory properties and because it is secreted in large amounts by both activated T-cells and keratinocytes from psoriatic lesions, is considered a major contributor to the pathologic changes seen in psoriatic skin such as keratinocyte hyperproliferation. Furthermore, one of the B7 family of receptors (the natural ligands for CD28 found on activated APC), BB1 has been shown to be expressed in psoriatic but not unaffected skin keratinocytes (Nickoloff et al (1993) *Am J Pathology* 142 : 1029-1040) underscoring the importance of T-cell activation in pathogenesis of the disease.

In other T-cell mediated skin disorders such as allergic contact dermatitis and lichen planus, CD28 was expressed in high levels in the majority of dermal and epidermal CD3<sup>+</sup> T-cells, but in normal skin and basal cell carcinoma (a non T-cell mediated skin disease), CD28 was expressed only in perivascular T-cells. Similarly, in both allergic contact dermatitis and lichen planus, B7 expression was found on dermal dendritic cells, dermal APCs and on keratinocytes, but not in normal skin and basal cell carcinoma (Simon et al (1994) *J Invest Derm* 103 : 539-543). Therefore this suggests that the CD28/B7 pathway is an important mediator of T-cell-mediated skin diseases.

Aberrant T-cell activation associated with certain autoimmune diseases caused by the loss of self-tolerance is predominantly characterized by the presence of CD28<sup>+</sup> T-cells and expression of its ligand, B7 on activated professional APCs (monocyte, macrophage or dendritic cells). These include autoimmune Graves thyroiditis (Garcia-Cozar et al (1993) *Immunologia* 12 32), sarcoidosis (Vandenberghe et al (1993) *Int Immunol* 5 : 317-321), rheumatoid arthritis (Verwilghen et al (1994) *J Immunol* 153 : 1378-1385) and systemic lupus erythematosus (Sfikakis et al (1994) *Clin Exp Immunol* 96 : 8-14). In normal T-cell activation, which mediates the rejection of transplanted cells and organs, the binding of CD28 by its appropriate B7 ligand during T-cell receptor engagement is critical for proper

allogeneic response to foreign antigens, for example, on donor tissue (Azuma et al (1992) *J Exp Med* 175 : 353-360, Turka et al (1992) *Proc Nat Acad Sci USA* 89 : 11102-11105).

Traditional therapies for autoimmune diseases do not prevent T-cell activation; the effector step in the autoreactive immune responses to self-antigen. Drugs, such as steroids and non-steroid anti-inflammatory drugs (NSAIDS), are currently used to ameliorate symptoms, but they do not prevent the progression of the disease. In addition, steroids can have side effects such as inducing osteoporosis, organ toxicity and diabetes, and can accelerate the cartilage degeneration process and cause so-called post-injection flares for up to 2 to 8 hours. NSAIDS can have gastrointestinal side effects and increase the risk of agranulocytosis and iatrogenic hepatitis.

Immunosuppressive drugs are also used as another form of therapy, especially in advanced disease stages. However, these drugs suppress the entire immune system and often treatment has severe side effects including hypertension and nephrotoxicity. Also established immunosuppressants such as cyclosporin and FK506 cannot inhibit the CD28-dependent T-cell activation pathway (June et al (1987) *Mol Cell Biol* 7 : 4472-4481).

Current agents which affect T-cell activation include synthetic peptides, monoclonal antibodies and soluble forms of T-cell activation molecules. To date competitive synthetic peptides to T-cell activation molecules such as CD28, CD40L and the CAM family of adhesion molecules have not been identified. Monoclonal antibodies (mAb) have been shown to have possible therapeutic effect in such T-cell mediated diseases such as psoriasis (anti-CD4 (Prinz et al (1994) *Lancet* 338 : 320-321)) and immunosuppression of normal T-cell activation in allografts (anti-VCAM-1 and VLA-4 (Isobe et al (1994) *J Immunol* 153 : 5810-5818)). However, with chronic treatment, the host animal develops antibodies against the monoclonal antibodies thereby limiting their usefulness. 'Humanized' monoclonal antibodies, have been developed which apparently reduce the risk of an induced immune response to these mAbs. However, these are still under development and in addition, these new mAbs remain large proteins and therefore may have difficulty reaching their target sites. Soluble forms of T-cell activation molecules such as CTLA-4Ig, containing the extracellular domain of the human CTLA-4 gene (which is sequentially related to CD28), fused to a human Ig C $\gamma$  chain, have been developed. CTLA-4Ig has been shown to specifically block normal T-cell activation by preventing rejection of xenogeneic (Lenschow et al (1992) *Science* 257 : 789-792) and allogeneic (Turka et al (1992) *Proc Nat Acad Sci USA* 89 : 11102-11105) cardiac allografts in rats and have therapeutic effect on aberrant T-cell activation such as found in rat autoimmune glomerulonephritis (Nishikawa et al (1994) *Eur J Immunol* 24 : 1249-1254). Soluble CTLA-4Ig however suffers from similar limitations as monoclonal

antibodies in addition to the expense of their production. Also the true function of this CD28-like molecule is not known therefore this needs to be fully determined before any therapeutic benefit can be evaluated.

Inhibition of the cell-surface expression of CD28 leads to prolonged unresponsiveness or deletion of activated T-cells. Inactivation prevents T-cell proliferation and arrest of T-cell-specific production of specific immunoregulatory cytokines such as interleukin-2, interferon-gamma and interleukin-8.

Regulation of CD28 gene expression can be achieved using antisense and triplex-forming oligonucleotides by hybridizing oligodeoxy-ribonucleotides or oligoribonucleotides to DNA or RNA sequences within the CD28 gene or promoter region (patent CD28 Tam). Oligonucleotides avoid many of the pitfalls of current agents used to block the effects of normal and abnormal T-cell activation. However, these oligos designed for antisense strategies are susceptible to degradation by intracellular nucleases or nucleases present in the extracellular milieu.

The binding of DNA (or RNA) to protein has been shown previously to be a fundamental pathway by which transcription of a gene is controlled. These regulatory proteins or transcription factors recognize DNA sequences with specific secondary structure and the ensuing interaction can lead to positive or negative control of gene expression. Aptamers are short oligonucleotide sequences which can specifically bind specific proteins. It has been demonstrated that different aptameric sequences can bind specifically to different proteins, for example, the sequence GGNNGG where N= guanosine (G), cytosine (C), adenosine (A) or thymidine (T) binds specifically to thrombin (Bock et al (1992) *Nature* **355**: 564-566 and patent #5582981 (1996) Toole et al).

Aptameric sequences have not been described, however, which can function as competitive inhibitors of DNA-binding sites on regulatory proteins known as transcription factors. Transcription factors are a class of proteins which regulate genes by primarily binding to specific regulatory sequences in the 5' upstream promoter region of those genes. This interaction leads to initiation of transcription. Certain transcription factors such as Sp1, AP2, AP-1, EGR-1 and NF $\kappa$ B are critical in the activation of T and B lymphocytes (Skerka et al *J Biol Chem* **270**: 22500-22506, Jung et al (1995) *Ann N Y Acad Sci* **766**: 245-252). In some cases these transcription factors are induced by signals initiated following costimulation (Jung et al (1995) *Ann N Y Acad Sci* **766**: 245-252). Thus, there is still a need to develop agents and methods for interfering with the interaction of protein with specific DNA binding sites which would lead to suppression of certain immune pathways including the costimulatory pathway.

## SUMMARY OF THE INVENTION

In accordance with the present invention, aptamer oligonucleotides are provided which were designed to specifically bind to the DNA binding site of proteins such as Sp1 and Sp1-related proteins which regulate the genes which encode costimulatory molecules such as CD28 and cytokines such as IL-2 and GMCSF.

In preferred embodiments, the oligonucleotides are designed to bind to specific regulatory proteins such as Sp1 and Sp1-related proteins and act to compete with the binding of these transcription factors to the promoter region of the genes which are under their control. This serves to modulate gene expression by preventing transcription of the gene. Thus the aptamer oligonucleotides are able to inhibit the function of RNA or DNA, either its translation into protein, its translocation into the cytoplasm or any other activity necessary to its overall biological function. The failure of the RNA or DNA to perform all or part of its function results in failure of a portion of the genome controlling T-cell activation to be properly expressed, thus modulating said metabolism.

It is preferred to target aptameric nucleic acid decoys to compete with the DNA-binding sites of regulatory proteins which specifically regulate molecules which can modulate T cell activation. It has been discovered that the CD28 protein is particularly useful for this approach. Inhibition of CD28 and CD28-related gene expression is expected to be useful for the treatment of psoriasis and other skin diseases, syndromes with aberrant T-cell activation, autoimmune disorders and allograft rejection.

Methods of modulating T-cell activation comprising contacting a patient with an oligonucleotide which competes with the DNA-binding site of a regulatory protein such as to inhibit expression of a regulated protein known to be capable of modulating T-cell activation are provided. Oligonucleotides which bind to proteins such as Sp1 and Sp1-related proteins which regulate transcription of CD28 and CD28-related genes are preferred.

In another aspect of the invention, aptamers are administered to provide therapies for diseases which involve aberrant T-cell activation such as psoriasis, AIDS-exacerbated psoriasis and other skin diseases, Type I (insulin-dependent) diabetes mellitus, thyroiditis, sarcoidosis, multiple sclerosis, autoimmune uveitis, rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease (Crohn's and ulcerative colitis), septic shock, tumor-induced cachexia and aplastic anemia and to regulate normal T-cell activation such as in allograft rejection. This can be achieved by perturbation in the synthesis and expression of T-cell activation molecules including CD28 and CD28-related molecules.

In yet another aspect of the invention, aptamers are provided which are capable of binding specific regulatory proteins such as Sp1 and Sp1-related proteins and thus inhibit

transcription of genes such as CD28 and CD28-related proteins which a) are normally regulated by these proteins and b) can modulate T cell responses.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIGURES 1A and 1B are graphical representations of the *in vitro* stability of  $^{32}\text{P}$ -labeled phosphorothioate, ICN 16064 (Seq #4), in extracellular fluid and in Jurkat cells, respectively. FIGURES 1C and 1D are graphical representations of the *in vitro* stability of  $^{32}\text{P}$ -labeled phosphorothioate, ICN 16214 (Seq #21), in extracellular fluid and in Jurkat cells, respectively.

FIGURES 1E and 1F are graphical representations of time-dependent degradation (0 - 96 h) of each oligonucleotide ICN 16064 (Seq #4) and ICN 16214 (Seq. #21), (2000 cpm) as assessed by electrophoresis on a 20% polyacrylamide denaturing gel followed by visualization using a PhosphorImager. The percentage of intact full length  $^{32}\text{P}$ - RT03S (○) and  $^{32}\text{P}$ - RTCO6S (●) remaining at each time point, relative to  $t = 0$ , was determined in eluates from 10000 cpm of extracellular (Figure 1E) and cell (Figure 1F) applied through Nickspin columns (Pharmacia). Molecular weight standards (Std),  $^{32}\text{P}$ -dNTP (N) and free  $^{32}\text{P}$ -orthophosphate (P) were simultaneously analysed.

FIGURE 2 is a graphical representation of a gel shift analysis which demonstrates that oligonucleotides containing a G-rich 12 mer sequence motif (lane 5 and 11) give a distinct band A which differs in electrophoretic shift to band B observed with other phosphorothioate oligonucleotides following incubation with HeLa nuclear extract. Band C is  $^{32}\text{P}$ -oligo alone.

FIGURE 3 is a graphical representation of chloramphenicol acetyltransferase (CAT) expression following the transfection of Jurkat cells with plasmid vectors containing a 226 bp insert from the CD28 promoter region (residues -197 to +28) (28b) or a mutant with a substitution at residues -51 to -22 with Seq#3 from Table 1, (28h-1) upstream of the CAT reporter gene, and following treatment with and without the phosphorothioate oligonucleotides, ICN16064 and ICN 16481.

FIGURE 4 is a graphical representation of a gel supershift assay showing that the binding of Sp1 to 28b, the upstream region -197 to +28 of the CD28 gene is specific.

FIGURE 5 is a graphical representation of the binding of Sp1 to the  $^{32}\text{P}$ -labeled double stranded oligo 28b (which is derived from the parent 28b - Seq #1 Table 1) and the competition binding of cold double stranded oligo 28b and the aptameric oligos FIGURE 1A and 16481.

## DETAILED DESCRIPTION OF SPECIFIC EMBODIMENTS

Aptameric oligonucleotides which specifically bind to the DNA-binding site of regulatory proteins such as Sp1 and Sp1-related proteins will prevent the binding of the regulatory protein with specific double-stranded region of DNA in the promoter region the gene of interest. The competitive binding by the aptamer would hinder transcription of the gene and thus inhibit the flow of genetic information from DNA to protein. The properties of oligonucleotides which make them specific for their target also make them versatile. Because oligonucleotides are long chains of four monomeric units they may be readily synthesized for any target RNA sequence.

Oligonucleotide-mediated inhibition of gene expression has been demonstrated in many model and *in vitro* systems and has therapeutic potential as a new strategy for treating many human diseases (Uhlmann and Peyman (1990) *Chem Rev* 90 : 544-584, Zon and Stec (1991) *Oligonucleotides and analogues - A Practical Approach*: 87-108, Miller et al (1981) *Biochem* 20 : 1874-1880, Orson et al (1991) *Nucleic Acid Res* 19 : 3435-3441, Helene and Toulme (1990) *Biochem Biophys Acta* 1049 : 99-125, Thierry and Ditschilo (1992) *Nucleic Acid Res* 20 : 5691-5698). Because of recent advances in synthesis of nuclease resistant oligonucleotides, including phosphorothioates Zon and Stec (1991) *Oligonucleotides and analogues - A Practical Approach*: 87-108 and phosphorothioate-3'-hydroxypropylamine (Tam et al (1994) *Nucleic Acid Res* 22 : 977-986), which exhibit enhanced cell uptake, it is now possible to consider the use of oligonucleotides as a novel form of therapeutics. Aptameric oligonucleotides targeting regulatory protein binding sites represent an alternative class of nucleic acid-based compounds and they offer an ideal solution to the problems encountered in prior art approaches. They are directly involved in the modulation of specific gene expression and so switch off target protein expression and not the competitive inhibition of soluble receptors to the target protein, an interaction which requires a complete understanding of the binding mechanisms and affinity of receptor-ligand interaction. Oligonucleotides are small molecules therefore do not encounter the same steric problems as large molecule inhibitors.

## DESCRIPTION OF TARGETS

Targets contemplated herein include molecules which can be regulated by transcription factors which play an essential role in initiating or maintaining an immune response. These include the costimulatory molecules such as CD28 and cytokines such as IL-2, GM-CSF and IFN $\gamma$ .



For therapeutics, an animal suspected of having a disease which can be treated by decreasing the expression of costimulatory molecules such as CD28 or CD28-related molecules can be treated by administering oligonucleotides in accordance with this invention. Oligonucleotides may be formulated in a pharmaceutical composition, which  
5 may include carriers, thickeners, diluents, buffers, preservatives, surface active agents, liposomes or lipid formulations and the like in addition to the oligonucleotide. Pharmaceutical compositions may also include one or more active ingredients such as antimicrobial agents, anti-inflammatory agents, anesthetics, and the like in addition to oligonucleotide.

10 The pharmaceutical composition may be administered in a number of ways depending on whether local or systemic treatment is desired, and on the area to be treated. Administration may be topically (including ophthalmically, vaginally, rectally, intranasally), orally, by inhalation, or parenterally, for example by intravenous drip, subcutaneous, intraperitoneal or intramuscular injection.

15 Formulations for topical administration may include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms or gloves may also be useful.

20 Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders may be desirable.

Formulations for parenteral administration may include sterile aqueous solutions which may contain buffers, liposomes diluents and other suitable additives.

25 Dosing is dependent on severity and responsiveness of the condition to be treated, but will normally be one or more doses per day, with course of treatment lasting from several days to several months or until a cure is effected or a diminution of disease state is achieved. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates.

30 In a preferred systemic application, the aptamers are to be administered intravenously in a dose of 5mg/kg once per day. In a preferred topical application, the aptamers are to be administered in a 1 - 5% solution once per day. In a preferred pulmonary application, the aptamers are to be administered in an aerosolized dose of 5mg once per day.

35 The present invention employs aptameric oligonucleotides for use in inhibition of the function of RNA and DNA corresponding to proteins capable of modulating T-cell activation. In the context of this invention, the term 'oligonucleotide' refers to an oligomer

or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes oligomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages as well as oligomers having non-naturally occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of properties such as, for example, enhanced cellular uptake and increased stability in the presence of nucleases.

The oligonucleotides in accordance with this invention preferably comprise from about 3 to about 50 nucleic acid base units. It is more preferred that such oligonucleotides comprise from about 8 and 30 nucleic acid base units, and still more preferred to have from about 12 and 22 nucleic acid base units. As will be appreciated, a nucleic acid base unit is a base-sugar combination suitably bound to an adjacent nucleic acid base unit through phosphodiester or the other bonds.

The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis may also be employed, however the actual synthesis of oligonucleotides are well within the talents of the routineer. It is also well known to use similar techniques to prepare other oligonucleotides such as <sup>32</sup>P-phosphorothioates and 3'-amine-phosphorothioates.

In accordance with this invention, persons of ordinary skill in the art will understand that messenger RNA identified by the open reading frames (ORFs) of the DNA from which they are transcribed includes not only the information from the ORFs of the DNA, but also associated ribonucleotides which form regions known to such persons as the 5'-untranslated, the 3'-untranslated region and intervening sequence ribonucleotides. Thus, oligonucleotides may be formulated in accordance with this invention which are targeted wholly or in part to these associated ribonucleotides as well as to the informational ribonucleotides. In preferred embodiments, the aptameric oligonucleotide interacts with the DNA-binding site of a regulatory protein such as Sp1 and Sp1-related proteins, and in doing so interrupt the expression of a gene encoding a protein involved in T-cell activation. In preferred embodiments, said proteins to be regulated are CD28 and all homologues of the CD28 molecule. Oligonucleotides comprising sequences containing at least two G-rich regions defined as a region of four nucleotides containing at least three guanosine (G) residues such as GGGG, GNNG, GGNG where N = A, C, G, U or T are preferred. Two such G-rich regions separated by at most 6 residues and preferably 4 or less residues are useful in the invention. A preferred sequence segment which may be useful in whole or in part is :



5'	3'	SEQ ID
TTG GAG GGG GTG GTG GGG		FIGURE 1A
GGG GAG GAG GGG CTG GAA		ICN 16481
GGG GTG GTG GGG		ICN 16525
TTG GAG GGG GAG GAG GGG		ICN 16475
TTG GAG GGG GAG GTG GGG		ICN 16479
GGG TTG GAG GGG GTG GTG GGG		ICN 16065

While the illustrated sequences are believed to be accurate, the present invention is directed to the correct sequences should errors be found. Oligonucleotides useful in the invention comprise one of these sequences, or part thereof. Thus, it is preferred to employ any of these oligonucleotides as set forth above, or any of the similar oligonucleotides which persons of ordinary skill in the art can prepare from knowledge of the preferred oligonucleotide targets for the modulation of the synthesis of T-cell activation molecules including CD28 and CD28-related molecules. The inhibition or modulation of production of the CD28 and/or CD28 homologues are expected to have significant therapeutic benefits in the treatment of disease. In order to assess the effectiveness of the compositions, an assay or series of assays is required.

## EXAMPLES

### Oligonucleotides

Oligodeoxynucleotides were synthesized on an automated DNA synthesizer (Applied Biosystems model 394) using standard phosphoramidite chemistry.  $\beta$ -cyanoethylphosphoramidites, synthesis reagents and CPG polystyrene columns were purchased from Applied Biosystems (ABI, Foster City, CA). 3'-Amino-Modifier C3 CPG columns were purchased from Glen Research (Sterling, VA). For phosphorothioate oligonucleotides, the standard oxidation bottle was replaced with tetraethylthiuram disulfide/acetonitrile, and the standard ABI phosphorothioate program was used for the stepwise addition of phosphorothioate linkages. After cleavage from the controlled pore glass column, the protecting groups were removed by treating the oligonucleotides with concentrated ammonium hydroxide at 55 °C for 8 hours. The oligonucleotides were purified by HPLC using a reverse phase semiprep C8 column (ABI). Following cleavage of the DMT protecting group, treatment with 80 % acetic acid and ethanol precipitation, the purity of the product was assessed by HPLC using an analytical C18 column

(Beckman, Fullerton, CA). All oligonucleotides of >90 % purity were lyophilized to dryness. Oligonucleotides were reconstituted in sterile deionized water (ICN, Costa Mesa), adjusted to 400  $\mu$ M following evaluation of OD<sub>260nm</sub>, aliquoted and stored at -20 °C prior to experimentation. In all cases, at least three batches of each oligonucleotide listed in Table 1 were used.

#### **In vitro oligonucleotide stability studies**

Temporal oligonucleotide stability analyses were performed as described previously (Tam et al (1994) *Nucleic Acid Res* 22 : 977-986). Oligonucleotide degradation profiles were assessed by electrophoresis and quantitated using Nickspin columns.

#### **Cell lines and T cell purification**

Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coat following Ficoll-Hypaque density gradient centrifugation of 60 ml blood from healthy donors. T-cells were then purified from the PBMCs using Lymphokwik lymphocyte isolation reagent specific for T-cells (LK-25T, One Lambda, Canoga Park CA). An average yield of 40 - 60 x 10<sup>6</sup> T-cells were then incubated overnight at 37 °C in 20 - 30 ml RPMI-AP5 (RPMI-1640 medium (ICN, Costa Mesa, CA) containing 20 mM HEPES buffer, pH 7.4, 5 % autologous plasma, 1 % L-glutamine, 1 % penicillin/streptomycin and 0.05 % 2-mercaptoethanol) to remove any contaminating adherent cells. In all experiments, T-cells were washed with RPMI-AP5 and then plated on 96-well microtitre plates at a cell concentration of 2 - 3 x 10<sup>6</sup> cells/ml.

The T-cell lymphoma cell line, Jurkat E6-1 (CD28<sup>+</sup>/CD4<sup>+</sup>) cells (152-TIB) were maintained in RPMI-10 (RPMI-1640 medium containing 20 mM HEPES buffer, pH 7.4, 10 % fetal calf serum (FCS) (Hyclone, Logan, UT), 1 % L-glutamine and 1 % penicillin/streptomycin).

#### **Mitogen-induced T-cell activation and oligonucleotide treatment**

Prior to the addition of human peripheral T-cells or T-cell lymphoma cell lines (0.2 - 0.3 x 10<sup>6</sup>), duplicate 96-well microtitre plates were pre-coated with purified anti-CD3 monoclonal antibody (mAb) (6.25 - 200 ng/well) (clone HIT 3a, Pharmingen, San Diego, CA) and washed twice with cold phosphate-buffered saline, pH 7.4 (PBS). Anti-CD3 mAb-treated T-cells were further activated by the addition of 2 ng phorbol 12-myristate 13-acetate (PMA) (Calbiochem, La Jolla, CA) and incubated for 48 h at 37 °C. Anti-CD3/PMA-activated T-cells were treated with 1 - 20  $\mu$ M CD28-specific and control oligonucleotides immediately

following activation and re-treated 24 h later. T-cells from one duplicate plate was used for immunofluorescence analysis and the 1A used for cytokine studies and the second plate was used for T-cell proliferation analysis.

## 5 Immunofluorescence studies

Following activation, 150  $\mu$ l cell supernatant from the first duplicate microplate was transferred to another microplate for analysis of cell-derived cytokine production. The remaining cells were washed twice with isotonic saline solution, pH 7.4 (Becton Dickinson, Mansfield, MA) and resuspended in 50  $\mu$ l isotonic saline solution and split into  
10 two samples. One sample aliquot was co-stained with either PE-CD28/FITC-CD4 mAb and non-specific fluorescence was assessed by staining the second aliquot with PE/FITC-labeled isotype-matched control monoclonal antibody. All fluorescence-labeled monoclonal antibodies were obtained from Becton Dickinson (San Jose, CA). Incubations were performed in the dark at 4 °C for 45 min using saturating mAb concentrations.  
15 Unincorporated label was removed by washing in PBS prior to the analysis with a FACScan flow cytometer (Becton Dickinson). Antigen density was indirectly determined in gated live cells and expressed as the mean channel of fluorescence (MCF). Surface expression of the CD4<sup>+</sup>-subset of cells stained with CD28 mAb was determined by subtracting the MCF of CD28<sup>+</sup> CD4<sup>+</sup> from the MCF of CD28<sup>-</sup> CD4<sup>+</sup> cells. The viability of control untreated and oligonucleotide-treated cells were determined in each batch of all oligonucleotides in multiple donors by staining with the vital dye, propidium iodide (5  $\mu$ g/ml final concentration). The percentage of live cells which excluded propidium iodide was determined by flow cytometry and was > 90 % (range 90 - 99 %) following treatment with all batches of all oligonucleotides at a dose range of 1 - 20  $\mu$ M.  
20

25

## Cytokine analyses

Cell-derived human cytokine concentrations were determined in cell Supernatants from the first duplicate microplate. Mitogen-induced changes in interleukin-2 (IL-2) levels were determined using a commercially available ELISA kit (R & D systems  
30 Quantikine kit, Minneapolis, MN) All ELISA results were expressed as pg/ml.

## Electrophoretic mobility shift analyses (EMSA)

Test oligonucleotides were labeled at the 5' end with [ $\gamma$ -32P]-ATP (ICN, Costa Mesa, CA) using T4 polynucleotide kinase as per manufacturers protocol (Gibco BRL, Gaithersburg,  
35 MD). 10 $\mu$ g of HeLa cell nuclear extract (Promega) was incubated with approximately 80,000 cpm of labeled oligonucleotide for 20 min at room temperature. The binding

reaction mixtures contained 10mM Tris-HCl (pH 7.5), 50mM NaCl, 0.5mM DTT, 0.5mM EDTA, 1mM MgCl<sub>2</sub>, 4% glycerol and 0.5µg of poly(dI.dC). DNA-protein complexes were resolved by electrophoresis through a 4% polyacrylamide gel containing 0.5x TBE buffer (50mM Tris, 45mM boric acid, 0.5mM EDTA) for approximately 3 hr at 100 V. The gel was dried and autoradiographed using PhosphorImager (Biorad, Richmond, CA).

#### cDNA preparation for DNase footprint assay and gel shift assay

The cDNAs (about 300 base pairs) used in the protein-DNA binding in DNase footprint assays and gel shift assays were isolated from plasmids pCAT3e 28b, pCAT3e 28h or pCAT3e 28h-1. 60 µg of each plasmid were digested with BglII, some being put first on agarose gel to check for linearity, the rest then phenol/sevag extracted, ethanol precipitated and then resuspended in water and digested with SacI. Again, a small portion was put on the gel to check if it's cut. (2 bands should appear now: a 4 kb band and a 300 b.p. band.) The rest was phenol/sevag extracted and ethanol precipitated. For the following dephosphorylation, DNA pellet was resuspended in a small volume of water (62 µl), 1 µl of 20 U/µl alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) and 7 µl of the 10X reaction buffer were added. After incubating the reaction mixture at 37°C for 1 hour, 7 µl of pH 8.0, 0.2M EGTA was added and the whole tube was heated at 65°C for 10 min. The whole 77 µl of the dephosphorylated DNA were put on 1% agarose gel to purify the 300 b.p. band using Qiaquick gel extraction kit from Qiagen (Santa Clarita, CA). Final volume of the purified 300 b.p. band was 70 µl and its concentration was calculated as follows:  $60 \mu\text{g} \times (300 \text{ b.p.} / 4300 \text{ b.p.}) = 4.2 \mu\text{g}$ , assuming 50% recovery after all these manipulations:  $2.1 \mu\text{g} / 70 \mu\text{l} = 30 \text{ ng}/\mu\text{l}$ . For each <sup>32</sup>P end-labeling reaction (kinasing), 5 µl to 7 µl of the purified 300 b.p. DNA was used.

#### Polyacrylamide gel preparation for gel shift assay

4% non-denaturing polyacrylamide gel solution in 0.5X TBE was prepared according to the Promega Gel Shift Assay Systems technical bulletin (4% acrylamide, 0.05% bisacrylamide, 2.5% glycerol, 0.5X TBE). A stock of 250 ml of the above gel solution was prepared, filtered and kept at 4°C. For each use, 12.5 µl of TEMED and 187.5 µl of 10% ammonium persulfate was added to every 25 ml of the stock 4% gel solution and poured into 16.5 cm X 16.5 cm X 0.75 mm glass plates. Gels were always allowed to polymerize overnight for optimal results. The gel was pre-run in 0.5X TBE buffer for 30 min at 100V before loading the samples.

#### Double stranded oligonucleotides formation and purification

The method used here was from "Antiparallel polypurine phosphorothioate oligonucleotides form stable triplexes with the rat  $\alpha 1(I)$  collagen gene promoter and inhibit transcription in cultured rat fibroblasts" by Jacob Joseph et al. in Nucleic Acids Research, 1997, Vol. 25, No. 11, 2182-2188. Equal amounts of complementary single strands were heated at 80°C for 5 min in 0.25M NaCl, followed by slow cooling to room temp. Annealed double-stranded oligonucleotides were purified by electrophoresis on a 6% non-denaturing (29:1) polyacrylamide gel, later cut out, "crushed and soaked," ethanol precipitated using the methods described in "Molecular cloning, a laboratory manual" by Sambrook, Fritsch & Maniatis. About 20 ng of d.s. oligo were used in each labeling reaction.

#### End $^{32}\text{P}$ labeling of DNA

150 to 200 ng of the 300 b.p. cDNA or 20 ng of the d.s. oligo were incubated with 10 $\mu\text{Ci}$  of [ $\gamma$ - $^{32}\text{P}$ ] ATP (4500Ci/mmol, ICN, Irvine, CA) and 10U of kinase and 1X kinase buffer (both from Promega) in a 10 $\mu\text{l}$  volume at 37°C for 1 hour, and purified on Centri Spin-10 column (Princeton Separation, Adelphia, NJ). About 80,000 - 100,000 cpm of kinased DNA was used in each gel shift reaction.

#### Gel shift assay

Proteins (nuclear extract or purified transcriptional factor) were incubated with 1X gel shift buffer (Promega, Madison, WI) at room temp for 5-10 min before kinased DNA was added and incubated for another 20-30 min at room temp. Samples were then loaded on the pre-run 4% non-denaturing gel. After about 3-4 hours run at 100V in 0.5X TBE, gel was dried on 2 pieces of Whatman papers and exposed in a phosphor-imager overnight.

#### Antibody gel supershift assay

Antibody to Sp1 (clone 1C6, Santa Cruz Biotechnologies, Santa Cruz, CA) was pre-incubated with purified Sp1 (Promega) for 1 hour before the  $^{32}\text{P}$ -labeled cDNA or oligo was added.

#### Competition gel shift assay

About 70-100 molar excess of non-labeled oligonucleotides (either single-stranded or double-stranded) were pre-incubated with protein at room temp for about 30 min before the  $^{32}\text{P}$ -labeled DNA was added.

### The construction of pCAT3e 28b, pCAT3e 28h, pCAT3e 28h-1

CD28 upstream cDNA (-197 to +28) was produced by RT PCR using Jurkat total RNA as template. This piece of cDNA was first cloned into a TA cloning vector PCR 2.1 (Invitrogen, Carlsbad, CA). The same cDNA was later subcloned into pCAT3e (Promega) by inserting into the XhoI-SacI site. pCAT3e 28h and pCAT3e 28h-1 are mutants of pCAT3e 28b in which -51 to -22 sequences were deleted and substituted by 15 other nucleotides.

### Transfection (transient expression)

One day before transfection, Jurkat cells were prepared in 2 or 3 T150s at 1:4 or 1:5 dilutions from 80-90% confluent cells. Just before transfection, all cells were pooled in one flask and counted (concentration should be around  $40 \times 10^4$  per ml.)  $11 \times 4 \times 10^6$  cells for 10 transfection reactions were spun down in 50 ml conical tubes. Cells were washed 1X with half of the original volume of PBS, then resuspended in 44 ml prewarmed fresh Jurkat media (90% RPMI 1640, 10% FBS, 1% L-glutamate, 1% penicillin/streptomycin) so final concentration was  $1 \times 10^6$ /ml. 4 ml of the cells was pipetted in each of the wells in 6-well plates. 2.5  $\mu$ l of 2 mg/ml plasmid (pCAT3e series) was pipetted in a 1.5 ml tube, 147.5  $\mu$ l RPMI 1640 medium (no serum, no antibiotics) was added, then 20  $\mu$ l of the Superfact reagent from Qiagen was added to the plasmid/medium solution, mix by pipetting up and down 5X, and allowed to sit at room temperature for 5-10 min. The transfection complex was added drop-wise to the cells in each well, gently swirling the plate to mix. The cells were incubated in a 37°C, 5% CO<sub>2</sub> incubator, and harvested for CAT assay after 24 hours. If oligos were to be added after transfection, 50  $\mu$ l of the stock 400  $\mu$ M oligo was added to the cells at the designated time, (1 hour after transfection) and cells were returned to the incubator.

### CAT assay

After 24 hours of incubation, cells were harvested by pipetting the cells from each well to 15 ml conical tubes, making sure to rinse well with cell media so no cells were left behind. They were spun at 2,000 rpms for 5 minutes at room temperature. Media was pipetted off. Each cell pellet was washed 3X with 2 ml PBS (PBS was added, vortexed, spun, media pipetted off). As much of the final PBS wash as possible was removed with a pipette tip. 400  $\mu$ l of 1X Reporter Lysis Buffer (Promega CAT Enzyme Assay System) was added to each cell pipette, and transferred to a 1.5 ml tube. The cell pellet was incubated in lysis buffer at room temp. for 30 min, vortexed occasionally. These tubes were heated at 60°C for 10 min at the end of 30 min incubation, then spun at room temp,

12,000 rpms, 2 min, supernatant (lysate) was pipetted to a fresh 1.5 ml tube. For each CAT assay reaction, 100  $\mu$ l of the lysate was used, the rest was frozen at  $-80^{\circ}\text{C}$ . Each CAT assay was set up as follows: 18.5  $\mu$ l of water was combined with 100  $\mu$ l of lysate, 5  $\mu$ l of 5 mg/ml n-Butyryl CoA (Promega) and 1.5  $\mu$ l of 0.1  $\mu\text{Ci}/\mu\text{l}$  chloramphenicol- $^{14}\text{C}$  (ICN) in a 1.5 ml tube (total volume 125  $\mu$ l) and incubated at  $37^{\circ}\text{C}$  for 1 hour. At the end of 1 hour, 300  $\mu$ l of Xylene (ICN) was added to each tube, vortexed vigorously for 5 sec, spun at full speed for 3 min at room temp., 280  $\mu$ l of the upper (xylene) phase was pipetted to a fresh tube. 100  $\mu$ l of 0.25M Tris, pH 8.0, was added to the 280  $\mu$ l xylene phase, vortexed, and spun as above. 200  $\mu$ l of the upper phase was pipetted to a scintillation vial, 5 ml of scintillation fluid was added, mixed by inversion, and samples counted in the scintillation counter.

#### **In vitro oligonucleotide stability extends the biological activity of phosphorothioate oligonucleotides.**

Modification of oligos with phosphorothioate internucleotide linkages can impart nuclease resistance and thus extend the *in vitro* bioactivity from 1 - 2h to 24h (Stein, (1993)*Science* 261: 1004-1012). Here, we demonstrated that the G-rich oligo, FIGURE 1A (Seq #4) had greater *in vitro* stability than a non-G-rich phosphorothioate, FIGURE 1B (Seq #21). In Figure 1A the electropherograms clearly show that, for both extracellular 1A (S) and cell 1B (L), considerably more intact  $^{32}\text{P}$ -labeled FIGURE 1A (Seq #4) than FIGURE 1B (Seq #21) remained following a 96h incubation with Jurkat cells. Consistent with this observation are the Nickspin column data (Figure 1B). Here, the percentage of intact oligo recovered from FIGURE 1A (Seq #4) after 96h was 54% (S) and 59% (L) and from FIGURE 1B (Seq #21) was 10% (S) and 34% (L). These data suggest that greater nuclease resistance is imparted purely by the presence of G-rich regions in FIGURE 1A (Seq #4) and this is presumably associated with the ability of this particular oligo to form folded secondary structures.

#### **Inhibition of functional CD28 expression and CD28-specific IL-2 production in activated human T-cells by aptameric oligonucleotides is dependent on a specific G-rich motif**

The relative inhibition of expression of CD28 and CD28-specific IL-2 production by phosphorothioate oligonucleotide sequence # 4 to 21 (5  $\mu\text{M}$ ) from Table 1 is shown in Table 2. Here, we examined the precise sequential requirements for the bioactivity of these aptameric oligonucleotides. Table 2 shows that inhibitory activity was sequence-dependent, in particular, relying on the presence of motif containing two G-quartets



separated by four bases (Seq # 5 - 8). These data suggest that the interaction of an oligo such as FIGURE 1A (Seq # 4) and its putative target, is dependent on a precise conformational requirement like that seen in a oligo-protein interaction rather than a nucleic acid : nucleic acid hybridization requirement (as found with antisense and antigene models).

#### **Oligonucleotides containing a specific 12mer sequence motif forms a specific protein oligo complex**

Figure 2 shows the electrophoretic mobility shift analysis of <sup>32</sup>P-labeled oligonucleotides preincubated with HeLa cell extract. The list of oligos in Table 3 includes two [FIGURE 1A (Seq #4) and ICN 16481 (Seq # 5)] which contain a 12 mer motif bearing two sets of G-tetrads separated by 4 nucleotides. The motif bearing oligos (lanes 5 and 11) were the only test oligos to give such an oligo-protein shift (Band A) distinct from other phosphorothioate oligos. These data suggest that a specific protein-oligo interaction occurs with oligos containing the 12mer motif.

#### **Inhibition of functional CD28 expression in activated human T-cells by aptameric oligonucleotides correlates with presence of specific oligo-protein complex**

Table 4 compares the inhibitory effect on both mitogen-induced CD28 expression and IL-2 production by certain phosphorothioate oligonucleotides at 5  $\mu$ M, with their aptameric ability to form a specific oligo-protein complex when incubated with HeLa nuclear extract, an enriched source of transcription factors. These data clearly indicate a correlation between the inhibitory activity of motif-bearing oligos on CD28 expression and IL-2 secretion and the formation of a specific gel shift band. Substitution within the 2 G-tetrads results in loss of function and results in the disappearance of the oligo-protein complex.

#### **The CD28 upstream promoter region -197 to + 28 (28b) binds Sp1**

<sup>32</sup>P-labeled CD28 promoter region -197 to +28 otherwise known as 28b was incubated with Sp1 protein and serial threefold dilutions of Sp1 antibody beginning with 0.5 $\mu$ g. A gel supershift assay was performed and the DNA-protein-antibody complexes resolved following electrophoresis and the data shown in Figure 4. The data shows that Sp1 does bind to 28b region of the CD28 promoter. This interaction is specific as following serial dilution of the specific Sp1 antibody to 0.00617 $\mu$ g the Sp1/<sup>32</sup>P-28b/Sp1 antibody band (band B) disappears leaving the 28b/Sp1 band (band A). This shows the 28b does specifically bind to Sp1. Free <sup>32</sup>P-labeled 28b is band C.



An oligo -51 to -22 derived from the CD28 upstream promoter region -197 to + 28 (28b) and contains the 12mer G-rich motif can also bind Sp1

In an effort to restrict the precise Sp1 binding region in 28b to the G-rich motif in the CD28 promoter region -197 to +28, we synthesized a double stranded (ds) 30mer oligo called 28b oligo (Seq #1 Table 1) which contains the 12mer GGGGAGGAGGGG within its sequence. We hypothesized that this was a Sp1 binding site in the CD28 promoter region. Following 32P-labeling, 28b oligo was incubated with Sp1 extract and indeed they bind to each other (Figure 5, band A, lane 2 and 3). Competition with cold ds 28b oligo caused the band to disappear showing indeed that the binding was specific to Sp1 (lane 4). Surprisingly, the single stranded phosphorothioate G-rich oligos, FIGURE 1A (lane 5) and 16481 (lane 6) (both which contain the G-rich motif) but not the control oligo ICN 16476 (lane 7) competed for binding to Sp1. This data shows that indeed the phosphorothioate G-rich oligos, FIGURE 1A and ICN 16481 can act as aptamers in binding to the DNA binding site of Sp1. The consequence of this interaction is the prevention of Sp1 binding to the Sp1 site at -51 to -22 in the promoter region and thus inhibit Sp1-mediated transcription of the CD28 gene and decrease expression of the mature CD28 protein.

Thus, aptamers and methods of modulating an immune response utilizing such aptamers have been disclosed. While specific embodiments have been disclosed herein, the scope of the invention is not be limited except through interpretation of the appended claims.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Robert Tam

(ii) TITLE OF INVENTION: G-RICH OLIGO APTAMERS AND  
METHODS OF MODULATING AN IMMUNE RESPONSE

(iii) NUMBER OF SEQUENCES: \_\_\_\_\_

## (iv) CORRESPONDENCE ADDRESS:

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## (v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: WordPerfect 6.1

## (vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: Not yet assigned
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- (C) CLASSIFICATION: Not yet assigned

## (viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Fish, Robert D.
- (B) REGISTRATION NUMBER: 33,880
- (C) REFERENCE/DOCKET NUMBER: 213/015

## (ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 714-525-3433
- (B) TELEFAX: 714-525-3303
- (C) TELEX:

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:  
GGGTTCCCTCG GGGAGGAGGG GCTGGAACCC

5 (3) INFORMATION FOR SEQ ID NO:2:  
    (i) SEQUENCE CHARACTERISTICS:  
        (A) LENGTH: 15 base pairs  
        (B) TYPE: nucleic acid  
        (C) STRANDEDNESS: Double  
10        (D) TOPOLOGY: unknown  
    (ii) MOLECULE TYPE: DNA (genomic)  
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:  
GGAGCACAGG GTGCT

15 (4) INFORMATION FOR SEQ ID NO:3:  
    (i) SEQUENCE CHARACTERISTICS:  
        (A) LENGTH: 15 base pairs  
        (B) TYPE: nucleic acid  
        (C) STRANDEDNESS: Double  
20        (D) TOPOLOGY: unknown  
    (ii) MOLECULE TYPE: DNA (genomic)  
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:  
TCATCACAGG GTGCT

25 (5) INFORMATION FOR SEQ ID NO:4:  
    (i) SEQUENCE CHARACTERISTICS:  
        (A) LENGTH: 18 base pairs  
        (B) TYPE: nucleic acid  
        (C) STRANDEDNESS: Double  
30        (D) TOPOLOGY: unknown  
    (ii) MOLECULE TYPE: DNA (genomic)  
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:  
TTGGAGGGGGG TGGTGGGG

35 (6) INFORMATION FOR SEQ ID NO:5:  
    (i) SEQUENCE CHARACTERISTICS:  
        (A) LENGTH: 18 base pairs  
        (B) TYPE: nucleic acid  
        (C) STRANDEDNESS: Double  
40        (D) TOPOLOGY: unknown  
    (ii) MOLECULE TYPE: DNA (genomic)  
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:  
GGGGAGGAGG GGCTGGAA

45 (7) INFORMATION FOR SEQ ID NO:6:  
    (i) SEQUENCE CHARACTERISTICS:  
        (A) LENGTH: 21 base pairs  
        (B) TYPE: nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

5 GGGTTGGAGG GGGTGGTGGG G

(8) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

15 TTGGAGGGGG AGGAGGGG

(9) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

25 TTGGAGGGGG AGGTGGGG

(10) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

35 TTGGAGGCCG TGGTGGCG

(11) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

45 TTGGAGCCGG TGGTGGCC

(12) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: unknown

5 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:  
TTGGAGGGGC TCCTCGGG

(13) INFORMATION FOR SEQ ID NO:12:

10 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: unknown

15 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:  
TTGGAGCCGG TGGTGG

(14) INFORMATION FOR SEQ ID NO:13:

20 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: unknown

25 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:  
GGGGTGGTGG GG

(15) INFORMATION FOR SEQ ID NO:14:

30 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: unknown

35 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:  
GGGGTTGGGG

(16) INFORMATION FOR SEQ ID NO:15:

40 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: unknown

45 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15  
TGGGG

(17) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16

GGGG

(18) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17

CACTGCGGGG AGGGCTGGGG

(19) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18

ATGGGGTGCA CAAACTGGGG

(20) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19

AACGTTGAGG GGCAT

(21) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20

TTCCAGCCCC TCCTCCCC

(22) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21

AACCTCCCCC ACCACCCC

(23) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22

ATTCGATCGG GGCGGGGCGA GC

(24) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23

CGCTTGATGA GTCAGCCGGA A

(25) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24

GATCGAACTG ACCGCCCCGCG GCCCCT

(26) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25  
AGTTGAGGGG ACTTTCCCAG GC

5 (27) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: Double

10 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26  
TGTCGAATGC AAATCACTAG AA

15 (28) INFORMATION FOR SEQ ID NO:27

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: Double

20 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27  
AGAGATTGCC TGACGTCAGA GAGCTAG

25 (29) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: Double

30 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28  
GCAGAGCATA TAAGGTGAGG TAGGA



Table 1.

Seq. No.	Oligo ID	Sequence
1	28b	5' GGG TTC CTC <u>GGG GAG GAG GGG</u> CTG GAA CCC 3' CCC AAG GAG CCC CTC CTC CCC GAC CTT GGG
2.	28h	5' GGA GCA CAG GGT GCT 3' CCT CGT GTC CCA CGA
3.	28h-1	5' TCA TCA CAG GGT GCT 3' AGT AGT GTC CCA CGA
4	ICN 16064	5' TTG GAG <u>GGG GTG GTG GGG</u>
5	ICN 16481	5' <u>GGG GAG GAG GGG</u> CTG GAA
6	ICN 16065	5' GGG TTG GAG <u>GGG GTG GTG GGG</u>
7	ICN 16475	5' TTG GAG <u>GGG GAG GAG GGG</u>
8	ICN 16479	5' TTG GAG <u>GGG GAG GTG GGG</u>
9	ICN 16480	5' TTG GAG <u>GCG GTG GTG GCG</u>
10	ICN 16538	5' TTG GAG <u>CCG GTG GTG GCC</u>
11	ICN 16539	5' TTG GAG <u>GGG CTC CTC GGG</u>
12	ICN 16523	5' TTG GAG <u>CCG GTG GTG G</u>
13	ICN 16525	5' <u>GGG GTG GTG GGG</u>
14	ICN 16526	5' <u>G GGG TTG GGG</u>
15	ICN 16483	5' <u>TG GGG</u>
16	ICN 16482	5' <u>G GGG</u>
17	ICN 16527	5' CAC TGC <u>GGG GAG GGC TGG GG</u>
18	ICN 16528	5' ATG <u>GGG TGC ACA AAC TGG GG</u>
19	ICN 16487	5' AAC GTT <u>GAG GGG</u> CAT
20	ICN 16476	5' TTC CAG CCC CTC CTC CCC
21	ICN 16214	5' AAC CTC CCC CAC CAC CCC
22	SP1	5' ATT CGA TCG <u>GGG CGG GGC</u> GAG C 3' TAA GCT AGC CCC GCC CCG CTC G
23	AP1 (c-jun)	5' CGC TTG ATG AGT CAG CCG GAA 3' GCG AAC TAC TCA GTC GGC CTT
24	AP2	5' GAT CGA ACT GAC CGC CCG CGG CCC CT 3' CTA GCT TGA CTG GCG GGC GCC GGG GA
25	NF-KB	5' AGT TGA <u>GGG GAC</u> TTT CCC AGG C 3' TCA ACT CCC CTG AAA GGG TCC G
26	OCT1	5' TGT CGA ATG CAA ATC ACT AGA A 3' ACA GCT TAC GTT TAG TGA TCT T
27	CREB	5' AGA GAT TGC CTG ACG TCA GAG AGC TAG 3' TCT CTA ACG GAC TGC AGT CTC TCG ATC
28	TFIID	5' GCA GAG CAT ATA AGG TGA GGT AGG A 3' CGT CTC GTA TAT TCC ACT CCA TCC T

Table 2. Identification of oligonucleotide sequence responsible for inhibition of CD28 expression and CD28-dependent IL-2 production

Oligo ID	Sequence	*Relative inhibition of	
		CD28	IL-2
ICN 16064	TTG GAG <u>GGGGTG</u> <u>GTGGGG</u>	100	100
ICN 16481	<u>GGGGAG</u> GAG <u>GGGCTG</u> GAA	100	100
ICN 16065	GGG TTG GAG <u>GGGGTG</u> <u>GTGGGG</u>	100	100
ICN 16475	TTG GAG <u>GGGGAG</u> <u>GAGGGG</u>	100	100
ICN 16479	TTG GAG <u>GGGGAG</u> <u>GTGGGG</u>	100	100
ICN 16480	TTG GAG <u>GCGGTG</u> <u>GTGGCG</u>	31	38
ICN 16538	TTG GAG <u>C CGGTG</u> <u>GTGGC C</u>	40	57
ICN 16539	TTG GAG <u>GGGCTC</u> <u>CTC GCG</u>	44	25
ICN 16523	TTG GAG <u>CCGGTG</u> <u>GTG G</u>	38	57
ICN 16525	<u>GGGGTG</u> <u>GTGGGG</u>	100	120
ICN 16526	<u>GGGG</u> <u>TTGGGG</u>	30	39
ICN 16483	<u>TGGGG</u>	2	2
ICN 16482	<u>GGGG</u>	2	2
ICN 16527	CAC TGC <u>GGGGAG</u> GGC <u>TGGGG</u>	58	76
ICN 16528	A <u>TGGGG</u> TGC ACA AAC <u>TGGGG</u>	51	63
ICN 16487	AAC GTT <u>GAGGGG</u> CAT	26	52
ICN 16476	TTC CAG CCC CTC CTC CCC	29	22
ICN 16214	AAC CTC CCC CAC CAC CCC	4	2

A 12 mer sequence containing two G quartets separated by four bases confers oligo activity. The minimal sequence required for in vitro activity of ICN 16064 was determined by the ability of sequential changes (in bold) to affect ICN 16064-mediated inhibition of CD28 expression in anti-CD3/PMA-activated human T cells and their effect on activated IL-2 production in Jurkat T cells.

\*Results are expressed relative to the activity of 5  $\mu$ M ICN 16064 (100%) whose range of inhibition in seven experiments was 52 - 79 % of CD28 expression and 76 - 89 % of IL-2 production.

**Table 3: Nuclear extract protein-binding profiles of phosphorothioate oligos (see Fig 2)**

<b>Oligo</b>	<b>Sequence</b>	<b>Lane No.</b>
ICN 16064	TTG GAG <u>GGG GTG GTG GGG</u>	11, 12
ICN 16481	<u>GGG GAG GAG GGG CTG GAA</u>	5, 6
ICN 16480	TTG GAG <u>GCG GTG GTG GCG</u>	7, 8
ICN 16538	TTG GAG <u>CCG GTG GTG GCC</u>	1, 2
ICN 16485	GTT GGA GAC CGG GGT TGG	3, 4
ICN 16476	TTC CAG CCC CTC CTC CCC	9, 10

Table 4: Inhibition of Functional CD28 Expression Correlates With the Presence of Specific Oligo-Protein Complex

Oligo	Sequence	Relative inhibition of expression (%)		Oligo/protein complex
		CD28	IL-2	
ICN16064	TTG GAG <u>GGG GTG GTG GGG</u>	100	100	Yes
ICN 16481	<u>GGG GAG GAG GGG CTG GAA</u>	100	100	Yes
ICN 16480	TTG GAG <u>GCG GTG GTG GCG</u>	31	38	No
ICN 16538	TTG GAG <u>CCG GTG GTG GCC</u>	40	57	No
ICN 16485	GTT GGA GAC CGG GGT TGG	11	15	No
ICN 16476	TTC CAG CCC CTC CTC CCC	29	22	No
ICN 16214	AAC CTC CCC CAC CAC CCC	4	2	No

Art 39  
Amend

30

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What is claimed is:

1. An aptamer having a sequence which includes at least two G-rich regions selected from the group of GGnG, GGGG, GnGG, nGGG and GGGn, where G is guanidine and n is any nucleotide.
2. The aptamer of claim 1 wherein at least two of the at least two regions are separated by less than two to seven nucleotides, inclusive.
3. The aptamer of claim 1 wherein at least two of the at least two regions are separated by three to six nucleotides, inclusive.
4. The aptamer of claim 1 wherein at least two of the at least two regions are separated by four nucleotides.
5. The aptamer of claim 1 which competes for a nucleic acid binding site of an immune regulatory protein.
6. The aptamer of claim 2 wherein the immune regulatory protein is selected from the group of SP1, NFkB, EGR1 and AP2.
7. The aptamer of claim 1 which competes for a nucleic acid binding site of an immune regulatory protein, wherein at least one of the at least two G-rich regions comprises GGnG, and at least two of the at least two regions are separated by two to seven nucleotides.
8. The aptamer of claim 1 which competes for a nucleic acid binding site of an immune regulatory protein, wherein at least one of the at least two G-rich regions comprises GGGG, and at least two of the at least two regions are separated by two to seven nucleotides, inclusive.
9. The aptamer of claim 1 which competes for a nucleic acid binding site of an immune regulatory protein, wherein at least one of the at least two G-rich regions comprises GnGG, and at least two of the at least two regions are separated by two to seven nucleotides, inclusive.

Art. 34  
Amend.

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10. The aptamer of claim 1 which competes for a nucleic acid binding site of an immune regulatory protein, wherein at least one of the at least two G-rich regions comprises nGGG or GGGn, and at least two of the at least two regions are separated by two to seven nucleotides, inclusive.
11. The aptamer of claim 1 comprising the sequence 5' TTG GAG GGG GTG GTG GGG. 3' (Seq. Id. No. 4).
12. The aptamer of claim 1 comprising the sequence 5' GGG GAG GAG GGG CTG GAA 3' (Seq. Id. No. 5).
13. The aptamer of claim 1 comprising the sequence 5' GGG GTG GTG GGG 3' (Seq. Id. No. 13).
14. The aptamer of claim 1 comprising the sequence 5' TTG GAG GGG GAG GAG GGG 3' (Seq. Id. No. 7).
15. The aptamer of claim 1 comprising the sequence 5' TTG GAG GGG GAG GTG GGG 3' (Seq. Id. No. 8).
16. The aptamer of claim 1 comprising the sequence 5' GGG TTG GAG GGG GTG GTG GGG 3' (Seq. Id. No. 6).
17. A method of modulating immune system response in a patient, comprising administering to the patient an aptamer according to any of claims 1-16.
18. A method of treating a patient having a condition characterized by an inappropriate immune system response, comprising administering to the patient an aptamer according to any of claims 1-16.
19. The method of claim 18 wherein the condition comprises a graft vs host response.
20. The method of claim 18 wherein the condition comprises an autoimmune disease.
21. The method of claim 20 wherein the condition comprises rheumatoid arthritis.

22. The method of claim 20 wherein the condition comprises multiple sclerosis.
23. The method of claim 20 wherein the condition comprises lupus erythematosus.
24. The method of claim 20 wherein the condition comprises insulin dependent diabetes mellitus.
25. The method of claim 20 wherein the condition comprises psoriasis.

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Figure 1A

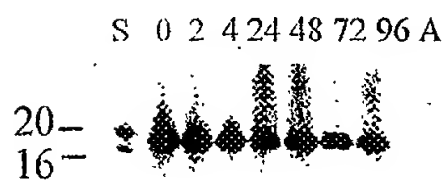
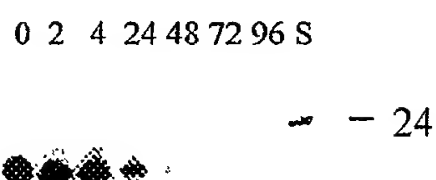


Figure 1C



N-

P-

Figure 1B

A 0 2 4 24 48 72 96



N-

N-

Figure 1D

A 0 2 4 24 48 72 96



P-

P-



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Figure 1E

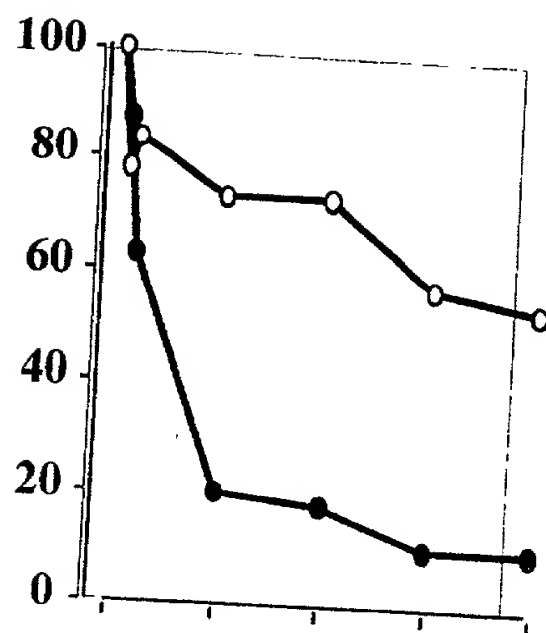
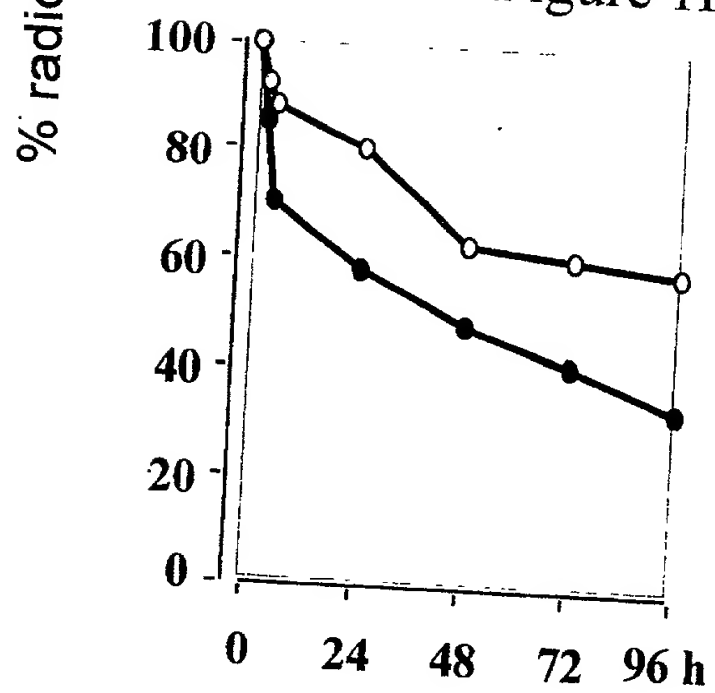
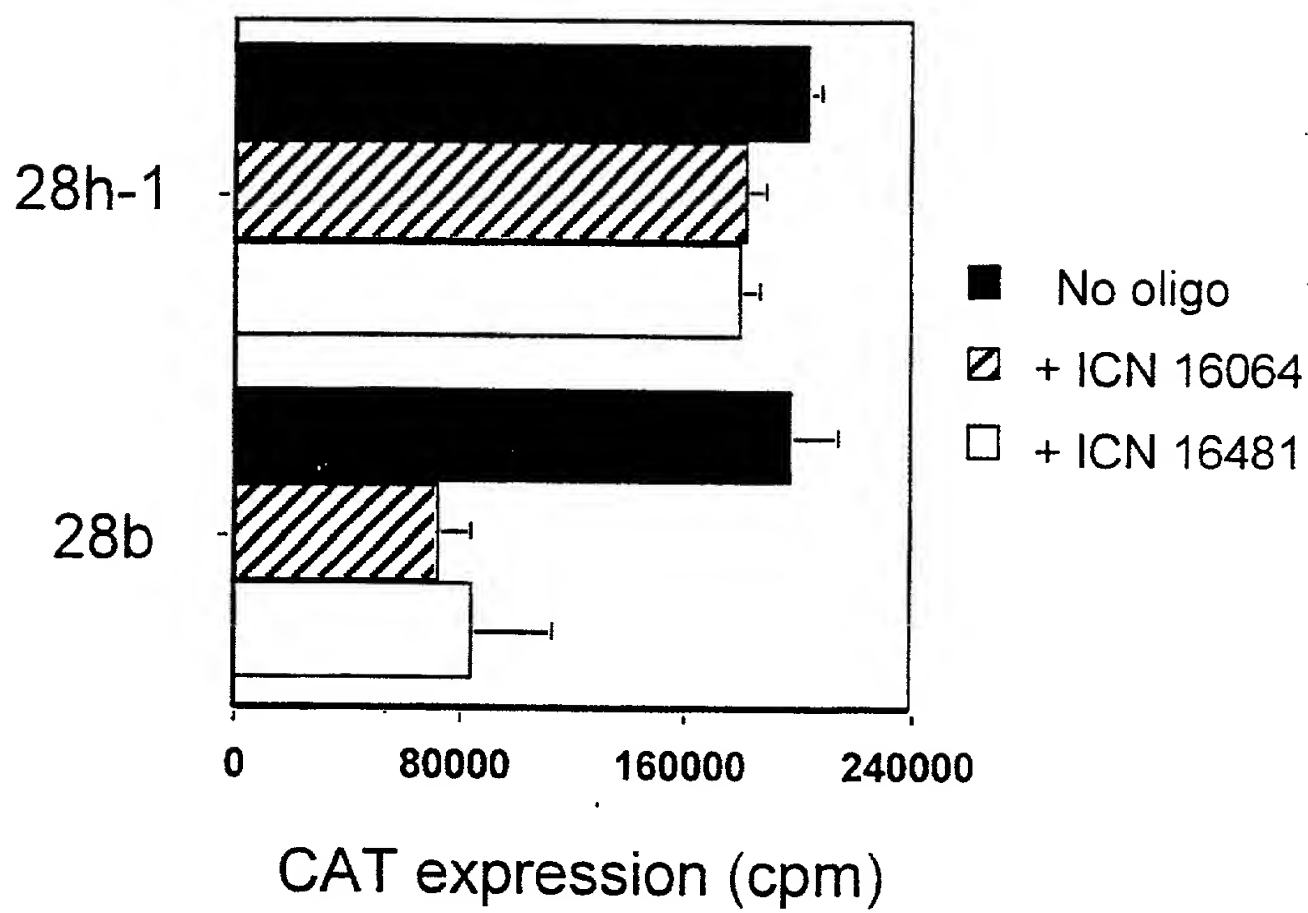


Figure 1F



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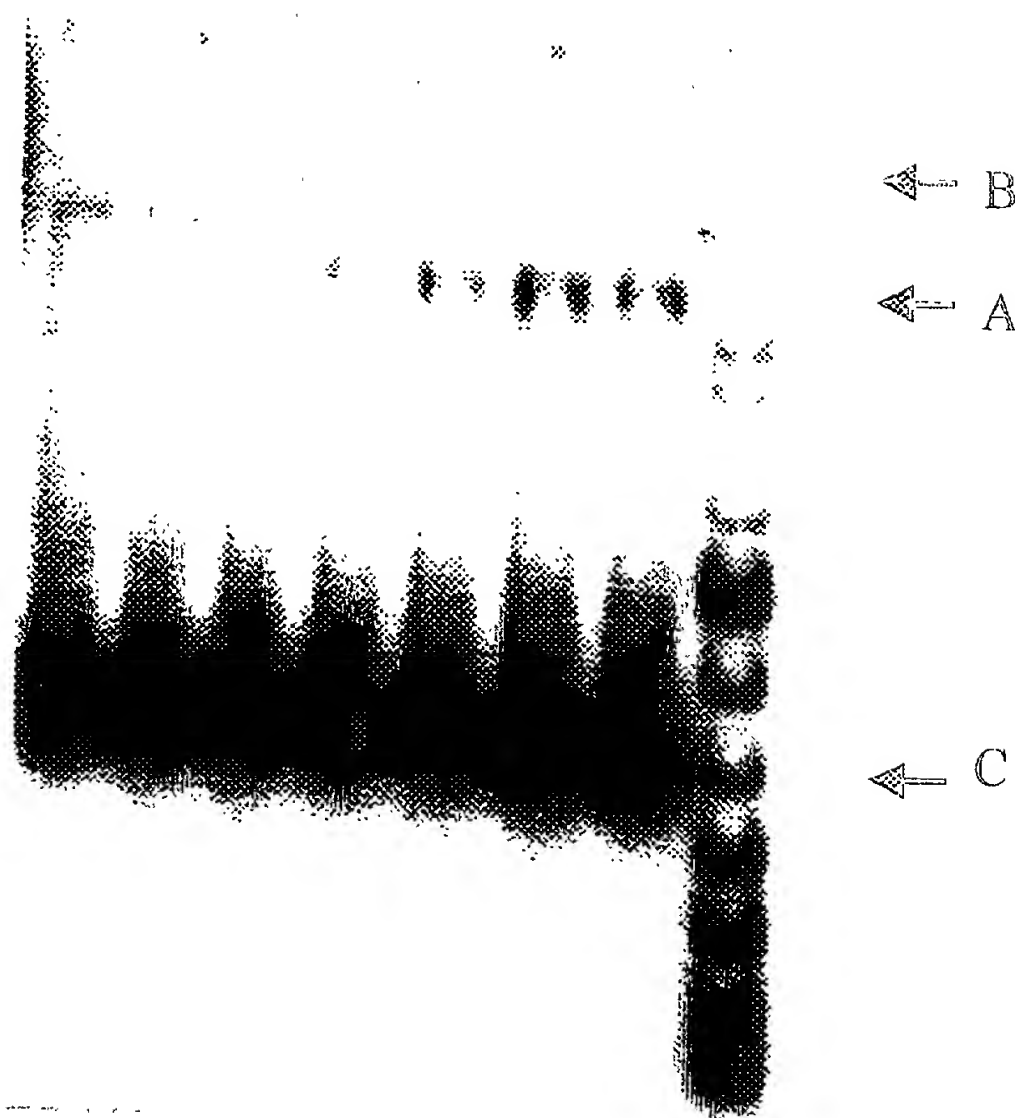
Figure 2



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Figure 3

Sp1 Ab dilution	1	3	9	27	81	243	0	
32P-28b	+	+	+	+	+	+	+	
Sp1 extract	+	+	+	+	+	+	+	
Lane no.	1	2	3	4	5	6	7	8



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Figure 4

1	2	3	4	5	6	7
+	+	+	+	+	+	+
-	+	+	+	+	+	+



Lane no.  
<sup>32</sup>P-28b oligo  
 Sp1 extract

← A

Lane 4 = Addition of cold ds 28b oligo  
 Lane 5 = Addition of cold ICN 16064  
 Lane 6 = Addition of cold ICN 16481  
 Lane 7 = Addition of cold ICN 16476

---

**COMBINED DECLARATION AND POWER OF ATTORNEY**

(ORIGINAL, DESIGN, NATIONAL STAGE OF PCT, SUPPLEMENTAL, DIVISIONAL,  
CONTINUATION, OR C-I-P)

---

As a below named inventor, I hereby declare that:

**TYPE OF DECLARATION**

This declaration is for a national stage of PCT application.

**INVENTORSHIP IDENTIFICATION**

My residence, post office address and citizenship are as stated below, next to my name. I believe that I am the original, first and sole inventor (*if only one name is listed below*) or an original, first and joint inventor (*if plural names are listed below*) of the subject matter that is claimed, and for which a patent is sought on the invention entitled:

**TITLE OF INVENTION**

G-rich Oligo Aptamers and Methods of Modulating an Immune Response

**SPECIFICATION IDENTIFICATION**

The specification was described and claimed in PCT International Application No. PCT/US97/23927 filed on December 19, 1997 and was amended under PCT Article 19 on June 16, 1999.

**ACKNOWLEDGMENT OF REVIEW OF PAPERS AND DUTY OF CANDOR**

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information, which is material to patentability as defined in 37, Code of Federal Regulations, § 1.56.

## POWER OF ATTORNEY

I hereby appoint the following practitioner(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Robert D. Fish

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Registration Number P-45,258

## AUTHORIZATION OF ATTORNEY(S) TO ACCEPT AND FOLLOW INSTRUCTIONS FROM REPRESENTATIVE

The undersigned to this declaration and power of practitioner hereby authorizes the U.S. practitioner(s) named herein to accept and follow instructions from

Bojan Cosic  
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as to any actions to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. practitioner(s) and the undersigned. In the event of a change in the person(s) from whom instructions may be taken, the U.S. practitioner(s) will be so notified by the undersigned.

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Robert D. Fish  
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## DECLARATION

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

## SIGNATURE(S)

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Inventor's signature

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